Title

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Journal

Clinical pharmacology and therapeutics, 100(5)

ISSN

0009-9236

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Publication Date

2016-11-01

DOI

10.1002/cpt.434

Peer reviewed

Metabolomic and Genome-wide Association Studies Reveal Potential Endogenous Biomarkers for OATP1B1

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Transporter-mediated drug-drug interactions (DDIs) are a major cause of drug toxicities. Using published genome-wide association studies (GWAS) of the human metabolome, we identified 20 metabolites associated with genetic variants in organic anion transporter, OATP1B1 ($P < 5 \times 10^{-8}$). Of these, 12 metabolites were significantly higher in plasma samples from volunteers dosed with the OATP1B1 inhibitor, cyclosporine (CSA) vs. placebo (q-value < 0.2). Conjugated bile acids and fatty acid dicarboxylates were among the metabolites discovered using both GWAS and CSA administration. *In vitro* studies confirmed tetradecanedioate (TDA) and hexadecanedioate (HDA) were novel substrates of OATP1B1 as well as OAT1 and OAT3. This study highlights the use of multiple datasets for the discovery of endogenous metabolites that represent potential *in vivo* biomarkers for transporter-mediated DDIs. Future studies are needed to determine whether these metabolites can serve as qualified biomarkers for organic anion transporters. Quantitative relationships between metabolite levels and modulation of transporters should be established.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Currently, regulatory agencies recommend the use of *in vitro* studies coupled with particular criteria to predict transporter-mediated clinical DDIs, and to trigger the conduct of expensive clinical DDI studies. Use of these assays and criteria results in many false-positive results.[49,50] Thus, endogenous biomarkers for membrane transporters are needed to better predict transporter-mediated clinical DDIs.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Can novel biomarkers for transporters be identified through multiscale approaches, including analysis of published genome-wide association studies of endogenous metabolites, metabolomic profiling of samples from clinical DDI studies, and uptake studies in transporter expressing cell lines?

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ Using multiscale approaches, we identified potential biomarkers of OATP1B1, which, if validated, may be used as probes for clinical DDIs. This is the first study showing that OATP1B1 interacts with fatty acid dicarboxylates.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

☑ Endogenous biomarkers, such as those identified in this study, can be used during drug development to better predict clinical DDIs.

Inhibition or induction of transporters in the liver, kidney, and intestine can result in clinical drug-drug interactions (DDIs). To predict whether a DDI is likely to occur and whether a specific clinical study is warranted, the US Food and Drug Administration, the European Medical Agency, and Japan's Pharmaceuticals and Medical Devices Agency have issued guidances that include a set of criteria based on cellular assays of drug transporter interactions.[1–3] Although, in general, *in vitro* cellular assays are good predictors of clinical DDIs, they are fairly conservative, erring on the side of excess false-positive over false-negative results. Endogenous metabolites that are selective substrates for one or a few drug transporters could be used during early clinical trials as biomarkers for potential clinical DDIs. These biomarkers could supplement *in vitro* studies and may reduce false-positive results from *in vitro* studies, as well as the cost and time required for clinical development.

For example, creatinine is a substrate of several organic cation and anion transporters in the kidneys, including organic cation transporter 2 (*SLC22A2*), multidrug and toxin extrusion protein 1 (*SLC47A1*), and organic anion transporter 2 (OAT2; *SLC22A7*).[4,5] A pharmaceutical agent that inhibits one or more of these transporters may cause an increase in serum creatinine

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Received 20 April 2016; accepted 15 July 2016; advance online publication 22 July 2016. doi:10.1002/cpt.434

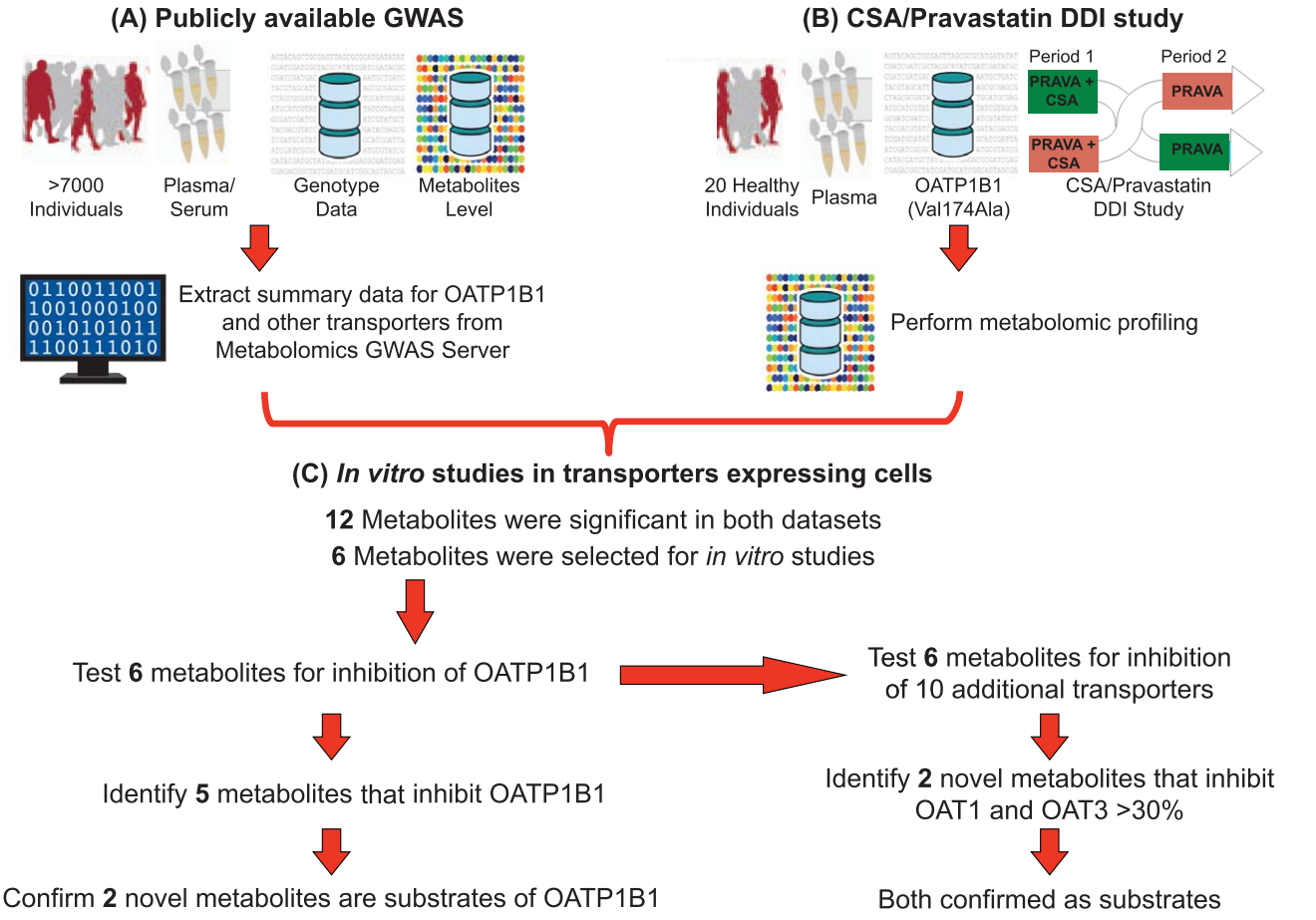

Figure 1 Schematic of the workflow applied to discovery and functional studies for identification of endogenous biomarkers for organic anion transporter, OATP1B1. This multiscale approach includes (**a**) using publicly available genome-wide association studies for human metabolites, (**b**) performing metabolomic profiling of a cyclosporine (CSA)/pravastatin drug-drug interaction (DDI) study, and (**c**) conducting *in vitro* studies in transporters expressing cells. SNPs within 25,000 bp up and downstream of 390 solute carrier (SLC) transporter genes were associated with 529 metabolites. In addition, plasma samples from 20 healthy volunteers taken from a CSA/pravastatin randomized crossover DDI study were used to profile metabolite levels. Twelve metabolites were significant in both datasets (see Results section). Transporter uptake and inhibition studies were performed to determine whether the selected six metabolites were inhibitors and/or substrates of OATP1B1 and 10 other selected transporters.

levels.[4,6] Thus, although not ideal because it is also a biomarker of kidney dysfunction, creatinine was suggested as a biomarker for renal transporter-mediated DDIs. Several metabolites that are more promising have been discovered in studies of transporter knockout mice and/or clinical studies of selected transporter-mediated DDIs. Examples include 6β-hydroxycortisol, N-methylnicotinamide, and bilirubin glucuronide, which are proposed as potential biomarkers of OAT3, multidrug and toxin extrusion protein 1/multidrug and toxin extrusion protein 2, and OATP1B1/OATP1B3/MRP2, respectively.[7–9] To date, there has been no systematic study to identify endogenous biomarkers of transporter-mediated DDIs.

The goal of this study was to identify endogenous metabolites for OATP1B1, which could serve as biomarkers of potential OATP1B1-mediated DDIs. Such metabolites could also provide valuable information about the biological function of the transporter. OATP1B1, a liver OAT, has been recognized by the International Transporter Consortium, the US Food and Drug Administration, and the European Medical Agency as an important protein in mediating DDIs.[10] Using data available from genome-wide association studies (GWAS) and clinical samples from a DDI study between pravastatin and cyclosporine (CSA), we discovered 12 metabolites that were significantly associated with genetic variants in OATP1B1 and exhibited increased plasma levels after administration of CSA, an inhibitor of OATP1B1. Following this multitiered approach, several of the 12 metabolites were found to be inhibitors or substrates of OATP1B1 in cell lines overexpressing the transporters (**Figure 1**). Collectively, our multiscale approach resulted in the identification of endogenous metabolites, which represent promising candidate biomarkers of DDIs mediated by OATP1B1.

RESULTS

GWAS from publicly available sources revealed novel endogenous metabolites for SLC transporters

The metabolomics GWAS server (http://mips.helmholtz-muenchen.de/proj/GWAS/gwas/index.php) allows access to genome-wide summary statistics for explorations of genetic loci influencing 529 human metabolites in up to 7,824 adult

individuals from two European ancestry cohorts.[11,12] **Supplementary Table S1** shows associations, with P values < 0.0001, for all the metabolites significantly associated with the single-nucleotide polymorphisms (SNPs) in SLC transporters. From the 390 SLC transporters extracted, 312 transporters were associated with at least one metabolite with P values < 0.0001. Several metabolites, which are known substrates of the SLC transporters, showed significant association with the SNPs within or near transporter genes (**Supplementary Table S2**). In addition, the association analyses showed many novel metabolites that have not been previously reported to be associated with particular transporters. For example, *SLC2A9*, a uric acid transporter,[13] which also transports fructose,[14] was strongly associated with plasma histidine levels in addition to uric acid levels. A second example is *SLC5A6*, a multivitamin transporter,[15] which was strongly associated with plasma mannose levels. **Supplementary Table S2** highlights the 41 SLC transporters that have at least one metabolite associated with it at genome-wide significance ($P < 5 \times 10^{-8}$).

OATP1B1 nonsynonymous variant, Val174Ala (c.521T>C, rs4149056), was associated with higher plasma levels of fatty acid dicarboxylic acids

The functional variant of OATP1B1, OATP1B1-Val174Ala (rs4149056), was significantly associated with 20 endogenous metabolites (**Supplementary Table S2**, **Supplementary Table S3**) in the genome-wide human metabolome study (**Figure 2a**). Among them are bile acids and steroids that have been reported previously to be substrates of OATP1B1.[16] The other metabolites, which showed significantly higher levels in individuals who were carriers of the OATP1B1-Val174Ala variant, were fatty acid dicarboxylates and lysolipids. Examples of fatty acid dicarboxylates were tetradecanedioate (TDA), hexadecanedioate (HDA), and octadecanedioate (ODA); examples of lysolipids with altered levels were 1-arachidonoylglycerophosphoethanolamine and 1-arachidonoylglycerophosphoinositol (**Supplementary Table S2**, **Supplementary Table S3**). Furthermore, the genome-wide association for some of these metabolites showed that OATP1B1 was the top association locus, for example, for the sulfated and glucuronidated bile acids and fatty acid dicarboxylates (**Figure 2a**). The levels of other known substrates of OATP1B1, such as bilirubin,[17] were also significantly associated with the OATP1B1 functional variant, however, at weaker P values ($P = 0.0009$). Notably, previous GWAS showed significant associations of OATP1B1 polymorphisms with bilirubin elevation,[18] although not all reached genome-wide significance.[19] In addition, other SNPs in OATP1B1 ($r^2 < 0.8$ based on the 1000 Genome Project European reference panel) were also associated with these 20 metabolites.

Cyclosporine, an OATP1B1 inhibitor, increased the plasma levels of 12 metabolites, which were associated with OATP1B1 Val174Ala in the GWAS

Plasma samples from 20 healthy volunteers obtained with and without CSA administration were evaluated for metabolite levels using the DiscoveryHD4 platform (Metabolon). The same technology, but with less coverage, was used in the study of Shin *et al*.[11] Among the 20 healthy subjects who self-reported their race as white, there were 10 volunteers who were homozygous for the reference allele of OATP1B1-Val174Ala, 7 volunteers who were heterozygous, and 3 who were homozygous for the Val174Ala variant allele. A total of 956 metabolites were identified, whereas 613 were metabolites of known structure and 343 were metabolites of unknown structural identity (**Supplementary Table S4**). Following log transformation and imputation of missing values (see **Supplementary Information**), we report a summary of 956 metabolites and their statistical significance P value (paired t test) and the estimate of the false discovery rate (q-value; **Supplementary Table S4**). Several metabolites were significantly higher in the CSA arm compared to the placebo arm ($P < 0.05$) and they belong to primary and secondary bile acids (sulfated and glucuronidated bile acids), eicosanoids, dicarboxylic acids, heme metabolites, and steroid sulfate and steroid glucuronide conjugates. Many of these metabolites are known OATP1B1 substrates.[16] In fact, these metabolites were also significant ($P < 0.05$) in the metabolomics GWAS server[10] (see **Table 1**). A total of 12 metabolites were significantly associated with variants in OATP1B1 when using both the GWAS data and the CSA inhibition data (**Figure 2**). That is, the levels of these 12 metabolites were significantly higher in individuals with the OATP1B1-Val174Ala (rs4149056) variant ($P < 0.05$) in the metabolomics GWAS server and in healthy volunteers following administration of CSA/pravastatin (False Discovery Rate q-value <0.2). Five of the 12 metabolites (X-11529, X-13429, TDA, HDA, and X-11905) also showed a relationship with the rs4149056 SNP in the 20 healthy volunteers following administration of the placebo ($P < 0.005$; **Figure 2b**). Other metabolites, including arginine, pro-hydroxy-pro, ergothioneine, and unknown metabolites (X-11440, X-14658, and X-11438) have weaker associations with SNPs in OATP1B1 ($P > 10^{-5}$) but no significant associations with OATP1B1-Val174Ala ($P > 0.05$). These metabolites were also not modulated upon CSA administration in the DDI study (False Discovery Rate >0.2). Among the 12 metabolites, fatty acid dicarboxylates (e.g., TDA and HDA) and 1,5-anhydroglucitol (1,5-AG) have not been associated with OATP1B1 previously. OATP1B1 was the top locus associated with 9 of the 12 metabolites. The functional variant OATP1B1-Val174Ala, or its linkage disequilibrium (LD) SNP ($r^2 > 0.8$), had the strongest association with eight of the nine metabolites (**Figure 2a and Supplementary Table S5**). In addition to the 12 metabolites that were significantly associated with *SLCO1B1* genotype using both methods, other metabolites were associated using one but not both of the methods. For example, lysolipids (e.g., 1-arachidonoylglycerophosphoinositol [$P = 3.7 \times 10^{-31}$]) were significantly associated with the *SLCO1B1* locus in the GWAS but not the CSA DDI study, perhaps because large samples would be needed in the clinical DDI study to detect differences in these metabolites. In contrast, these lysolipids may not be substrates of OATP1B1, but their metabolic products may be substrates of the transporter. Despite the fact that previous studies have shown that total bilirubin levels (conjugated and unconjugated) were elevated in the presence of an OATP1B1 inhibitor

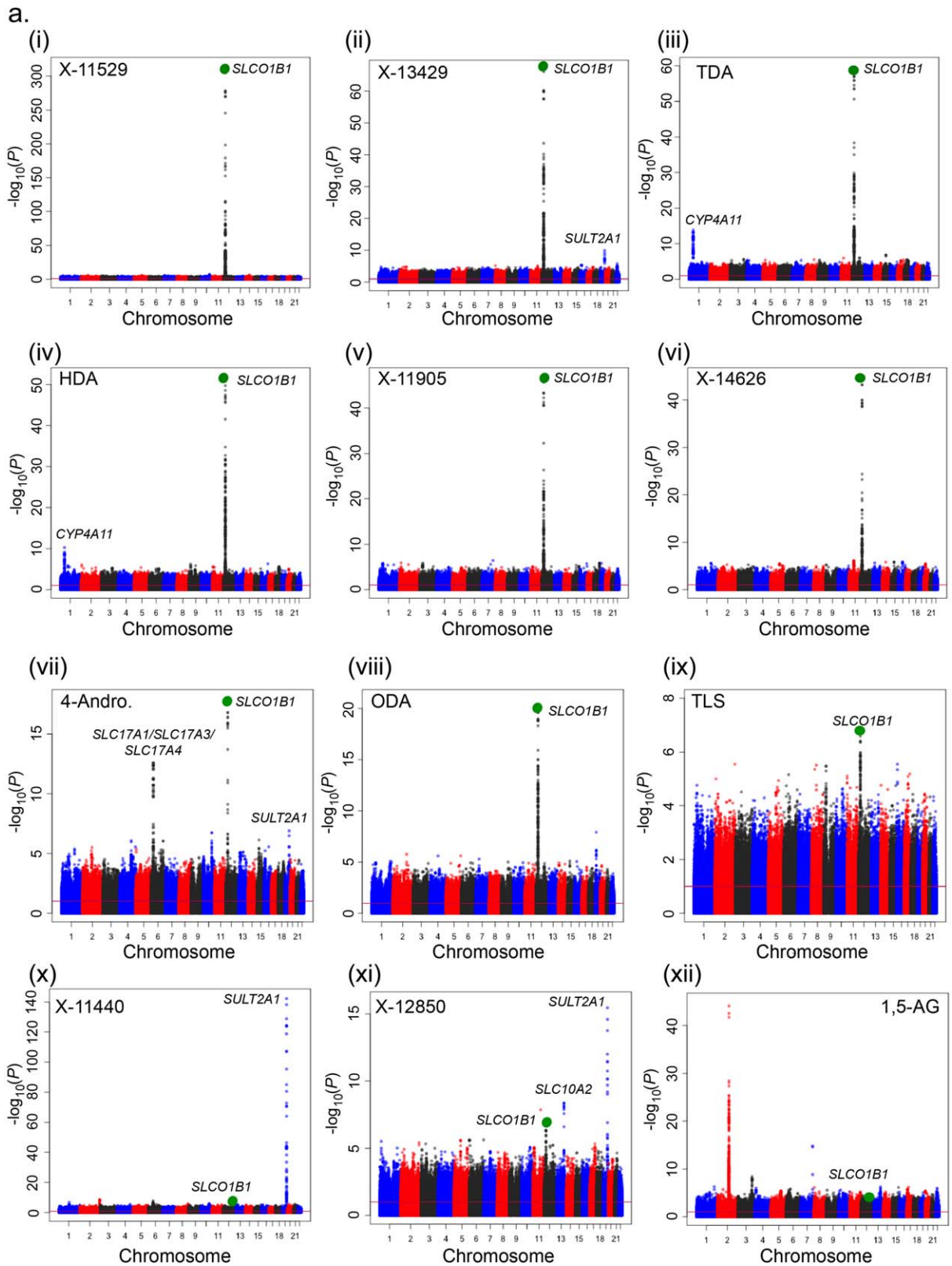
a.
(i)
X-11529
SLCO1B1
(ii)
X-13429
SLCO1B1
SULT2A1
(iii)
TDA
SLCO1B1
CYP4A11
(iv)
HDA
SLCO1B1
CYP4A11
(v)
X-11905
SLCO1B1
(vi)
X-14626
SLCO1B1
(vii)
4-Andro.
SLCO1B1
SLC17A1/SLC17A3/
SLC17A4
SULT2A1
(viii)
ODA
SLCO1B1
(ix)
TLS
SLCO1B1
(x)
X-11440
SULT2A1
SLCO1B1
(xi)
X-12850
SULT2A1
SLC10A2
SLCO1B1
(xii)
1,5-AG
SLCO1B1
-log10(P)
Chromosome

Figure 2.

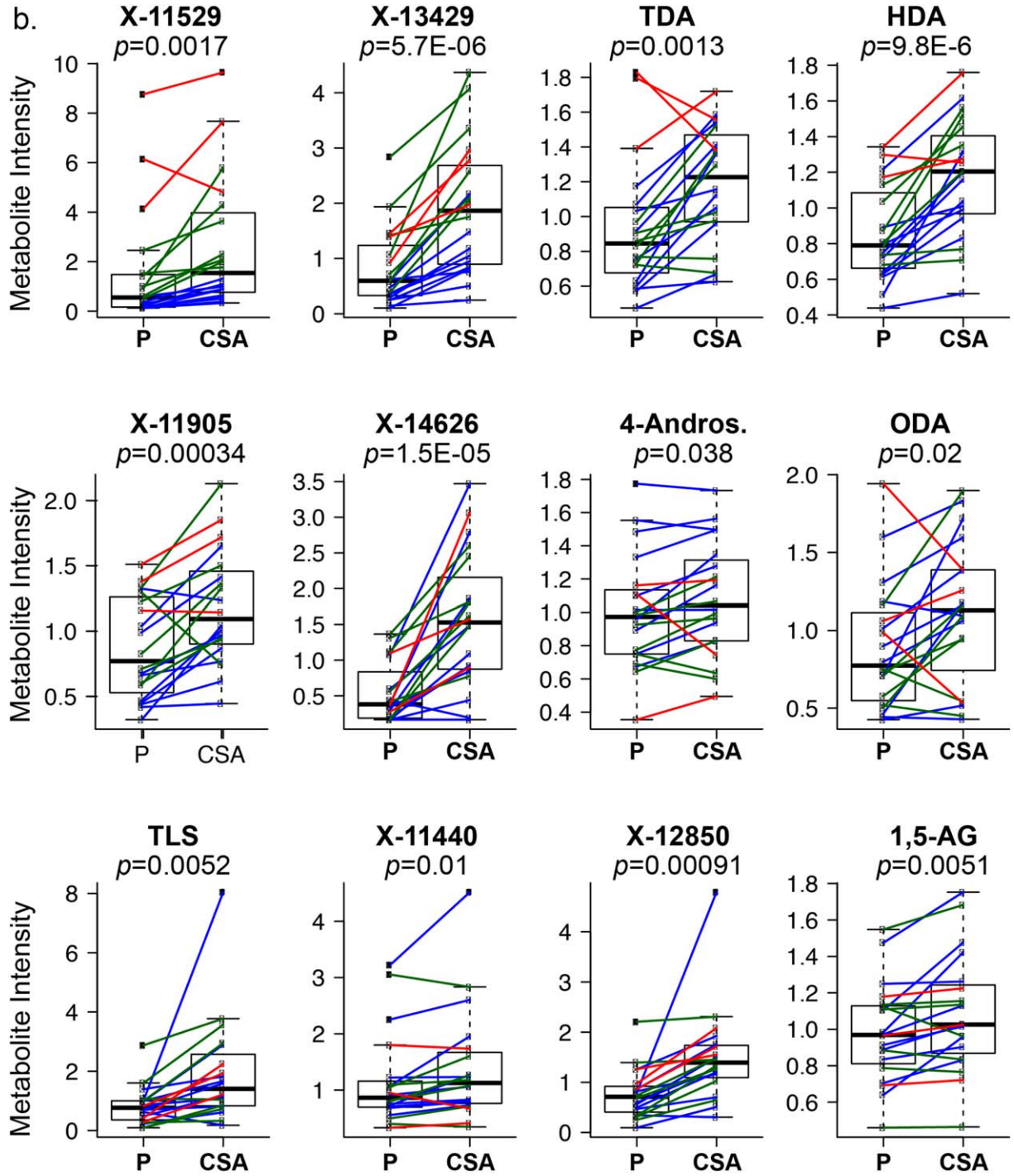

Figure 2 Twelve metabolites (i to xii) significantly associated with organic anion transporter OATP1B1-Val174Ala in both a genome-wide association study of human metabolites[11] and significantly associated in healthy volunteers receiving pravastatin and cyclosporine, an OATP1B1 inhibitor. (**a)** Manhattan plots of the 12 metabolites. Manhattan plots were generated using the publicly available data, which provided the meta-analysis *P* values of each single-nucleotide polymorphism (SNP; http://mips.helmholtz-muenchen.de/proj/GWAS/gwas/index.php?task=download). The large green circle represents the OATP1B1 functional variant, Val174Ala (rs4149056). Other loci, which include genes for transporters or enzymes, are labeled. OATP1B1-Val174Ala or its linkage disequilibrium, variant ($r^2 > 0.8$) is significantly associated with eight metabolites with the strongest *P* values in the genome-wide association study (GWAS; metabolites i–vii and ix). (**b**) The 12 metabolites significantly higher in healthy volunteers after administration of cyclosporine (CSA) compared to placebo (P). Each dot represents the metabolite intensity in the plasma from an individual after administration of placebo (P) or cyclosporine (CSA) and each line connects the metabolite intensity from the same individual following placebo or CSA administration. The color of the line represents the genotype of OATP1B1-Val174Ala, whereas blue represents homozygous reference allele, green represents heterozygous reference allele, and red represents homozygous-reduced function variant. The *P* values of the paired *t* test between the placebo and CSA treatment group are shown in the figure. Individuals with the variant for OATP1B1-Val174Ala (after placebo administration) had significantly higher metabolites levels of X-11529, X-13429, tetradecanedioate (TDA), hexadecanedioate (HDA), and X-11905 ($P < 0.005$). The mean and SD for each of the 12 metabolites in each genotype group are shown in **Supplementary Table S6**. Note: X-11529, glycochenodeoxycholate glucuronide; X-13429, glycodeoxycholate sulfate or isomer; X-11905, unknown; X-14626, unknown; 4-Andros: 4-androsten-3-beta, 17-beta-diol disulfate 2′; ODA, octadecanedioate; TLS, taurolithocholate 3-sulfate; X-11440, unknown; X-12850, glycochenodeoxycholate sulfate or isomer; 1,5-AG, 1,5-anhydroglucitol.

Table 1 Twelve candidate metabolites, which are significantly associated with higher levels in subjects with minor allele C for rs4149056 (OATP1B1-Val174Ala) with $P < 0.05$ and also significantly associated with higher levels in healthy volunteers (20 participants) after dosing with CSA with q <0.2

Metabolite name	Pathway	GWAS			Clinical DDI study			
		Effect[a]	SE	*P* value[b]	Median (placebo)	Median (CSA)	*P* value	Q value
X-11529 (likely to be glycochenodeoxycholate glucuronide)	Secondary bile acid metabolism	-0.30	0.0078	5.5E-315	0.55	1.54	0.0017	0.083
X-13429 (likely to be glycodeoxycholate sulfate or isomer)	Secondary bile acid metabolism	-0.14	0.0079	2.8E-67	0.60	1.87	5.74E-06	0.0027
TDA	Fatty acid, dicarboxylate	-0.099	0.0061	3.4E-59	0.85	1.23	0.0013	0.067
HDA	Fatty acid, dicarboxylate	-0.088	0.0058	1.7E-51	0.79	1.20	9.75E-06	0.0029
X-11905	Unknown	-0.083	0.0057	1.1E-47	0.77	1.09	0.00034	0.027
X-14626	Unknown	-0.053	0.0038	6.9E-44	0.38	1.53	1.53E-05	0.0029
4-Androsten-3beta,17beta-diol disulfate 2*	Steroid	-0.049	0.0055	1.3E-18	0.92[c]	1.17[c]	0.038[c]	0.18[c]
ODA	Fatty acid, dicarboxylate	-0.029	0.005	1.0E-08	0.74[c]	1.10[c]	0.0013[c]	0.08[c]
Taurolithocholate 3-sulfate	Secondary bile acid metabolism	-0.041	0.0078	1.5E-07	0.77	1.41	0.0052	0.18
X-11440	Unknown	-0.025	0.0064	0.00011	0.81[c]	1.18[c]	0.0048[c]	0.18[c]
X-12850 (likely to be glycochenodeoxycholate sulfate)	Secondary bile acid metabolism	-0.019	0.0071	0.0084	0.71	1.39	0.00091	0.055
1,5-AG	Glycolysis, gluconeogenesis, and pyruvate metabolism	-0.0078	0.0035	0.024	0.97	1.03	0.0051	0.18

1,5-AG, 1,5-anhydroglucitol; CSA, cyclosporine; GWAS, genome-wide association study; DDI, drug-drug interaction; HDA, hexadecanedioate; ODA, octadecanedioate; TDA, tetradecanedioate. 2*, The sulfated species could be in different site and the exact location may not be known.
[a]Effect: A negative effect means that the major reference allele (T) has lower levels of the metabolites. The negative effects were observed in two independent cohorts as described by Shin *et al*.[11] (2014). The effect for each cohort is available in **Supplementary Table S3**. [b]This is the meta-analysis *P* value. The meta-analysis was performed using two independent cohorts, as described by Shin *et al*.[11] (2014). The *P* value for each cohort is available in **Supplementary Table S2**. [c]The reported metabolite levels, *P* values, and q values after removing individuals who are homozygous for reduced function variant of OATP1B1-Val174Ala.
Note: **Figure 2a** shows the Manhattan plot for each of the metabolites above and **Figure 2b** shows the boxplot for each of the metabolites in healthy volunteers after administration of placebo or CSA.

(rifampicin or CSA at higher dose),[9,20,21] we did not observe significantly elevated levels of unconjugated bilirubin in our 20 healthy volunteers ($P < 0.2$). Our study may not have been sufficiently powered to detect the increased bilirubin levels due to its smaller effect size (as shown in **Supplementary Table S3**) or sample collection times may not have been optimal for observing changes in bilirubin levels.

Fatty acid dicarboxylates interacted with OATP1B1, but not OATP1B3, OATP1A2, and OATP2B1

Seven of the 12 metabolites identified from the GWAS and which also showed increased levels following CSA treatment (**Figure 1**, **Table 1**), six were tested in the follow-up *in vitro* studies: two sulfated and one glucuronidated bile acid were not available, so we tested the two parent compounds, glycochenodeoxycholate and glycodeoxycholate, bearing in mind that the results could be different for the conjugated metabolites (**Table 2**). Among the five metabolites that were not followed up *in vitro*, were three unknown metabolites (X-11905, X-14626, and X-11440), and two named biochemical (octanedioic acid and 4-androsten-3beta, 17beta-diol disulfate) that were not readily available for purchase.

We approached the *in vitro* studies by first testing the metabolites (at 30 μM) as competitive inhibitors of OATP1B1 and

Table 2 Inhibitor effect on 11 transporters using transfected cell lines or membrane vesicles from transfected cell lines

Metabolite name	Actual compound tested	OATP1B1	OATP1B3	OATP1A2	OATP2B1	OAT1	OAT2	OAT3	NTCP	ABCC2	ABCG2	ABCB11
X-11529 (likely glycochenodeoxycholate glucuronide)	Glycochenodeoxycholate											
X-12850 (likely glycochenodeoxycholate sulfate or isomer)												
X-13429 (likely glycodeoxycholate sulfate or isomer)	Glycodeoxycholate											
TDA	TDA											
HDA	HDA											
Taurolithocholate 3-sulfate	Taurolithocholate 3-sulfate											
1,5-AG	1,5-AG											
CSA	CSA									Ref. 51	Ref. 52	Ref. 53

≤30% inhibition >30–70% inhibition >70% inhibition

1,5-AG, 1,5-anhydroglucitol; CSA, cyclosporine; DDI, drug-drug interaction; HDA, hexadecanedioate; OAT, organic anion transporter; TDA, tetradecanedioate.
Metabolites were tested as inhibitors at 30 μM. The colors indicate the different range of inhibition by the metabolites. Since the publication of Shin *et al.*[11] in 2014, a few previously unknown compounds have been identified by Metablon, X-11529, X-13429, and X-12850, which are likely to be glycochenodeoxycholate glucuronide, glycodeoxycholate sulfate, and glycochenodeoxycholate sulfate, respectively.

other OATs (**Table 2**). Any metabolite that inhibited >30% was deemed an inhibitor of that transporter and was tested as a potential substrate. This method is accurate for identifying true inhibitors and substrates, although low potency inhibitors that may also be substrates of the various transporters would be missed. Among the six metabolites that were tested as inhibitors of OATP1B1 and other organic anion-transporting polypeptides (OATPs), OATP1B3, OATP1A2, and OATP2B1 in transfected cell lines (**Table 1**, **Figure 1**), OATP1B1 transporter activity was inhibited >70% at 30 μM by five of the metabolites (**Table 2**). In addition, we showed that TDA and HDA inhibited OATP1B1 potently (IC_{50} 1.6–4 μM; **Figure 3a and 3b**). Interestingly, substrate uptake by other OATPs, OATP1B3, OATP1A2, and OATP2B1 was not inhibited by TDA and HDA (**Table 2**). However, the other three metabolites (glycochenodeoxycholate, glycodeoxycholate, and taurolithocholate-3-sulfate) inhibited OATP1B3 and OATP2B1 >30% at 30 μM (**Table 2**). CSA inhibited several transporters, including OATP1B1, OATP1B3, Na+-taurocholate co-transporting polypeptide (NTCP), ABCC2, ABCB11, and ABCG2 (**Table 2**).

Because TDA, HDA, and 1,5-AG represent novel classes of metabolites not known previously to interact with OATP1B1, we tested them as substrates of the OATP1B1 in transfected cells. TDA and HDA showed significantly higher uptake in HEK293 cells overexpressing OATP1B1 compared with cells transfected with empty vector (EV; **Figure 3c**). Uptake of TDA (180 nM) and HDA (12 nM) was significantly inhibited by CSA (10 μM; **Figure 3c**). In addition, cells overexpressing OATP1B1-Val174Ala had intermediate uptake of TDA and HDA compared with cells containing wild type OATP1B1 or EV (**Figure 3d and 3e**). The 1,5-AG was not transported by OATP1B1 (**Supplementary Figure S1**) and also did not inhibit OATP1B1 at 30 μM (**Table 2**). Because SNPs within the OATP1B1 locus are in linkage disequilibrium with SNPs within or near to the genes encoding OATP1A2 and OATP1B3, and that they share overlapping substrates, thus, we evaluated the uptake of TDA and HDA in cells overexpressing these transporters and OATP2B1. We did not observe significant uptake of TDA and HDA in OATP1A2, OATP1B3, and OATP2B1 transfected cells (**Supplementary Figure S2**).

Fatty acid dicarboxylates interacted with organic anion transporters, OAT1 and OAT3

Primary and secondary bile acids, sulfated and glucuronidated bile acids, steroids, and conjugates of these metabolites are substrates of many transporters, including organic anion transporters (OAT1, OAT2, and OAT3), NTCP, ABCC2, ABCG2, and ABCB11.[22,23] Nothing has been reported for the dicarboxylic acids TDA and HDA, or 1,5-AG. Accordingly, we first screened the six metabolites as inhibitors of these seven transporters (**Table 2**). TDA and HDA inhibited OAT3 (>70%) and OAT1 (>30%) but not the other transporters. The other metabolites had different inhibition characteristics, with 1,5-AG not acting as an inhibitor (**Table 2**).

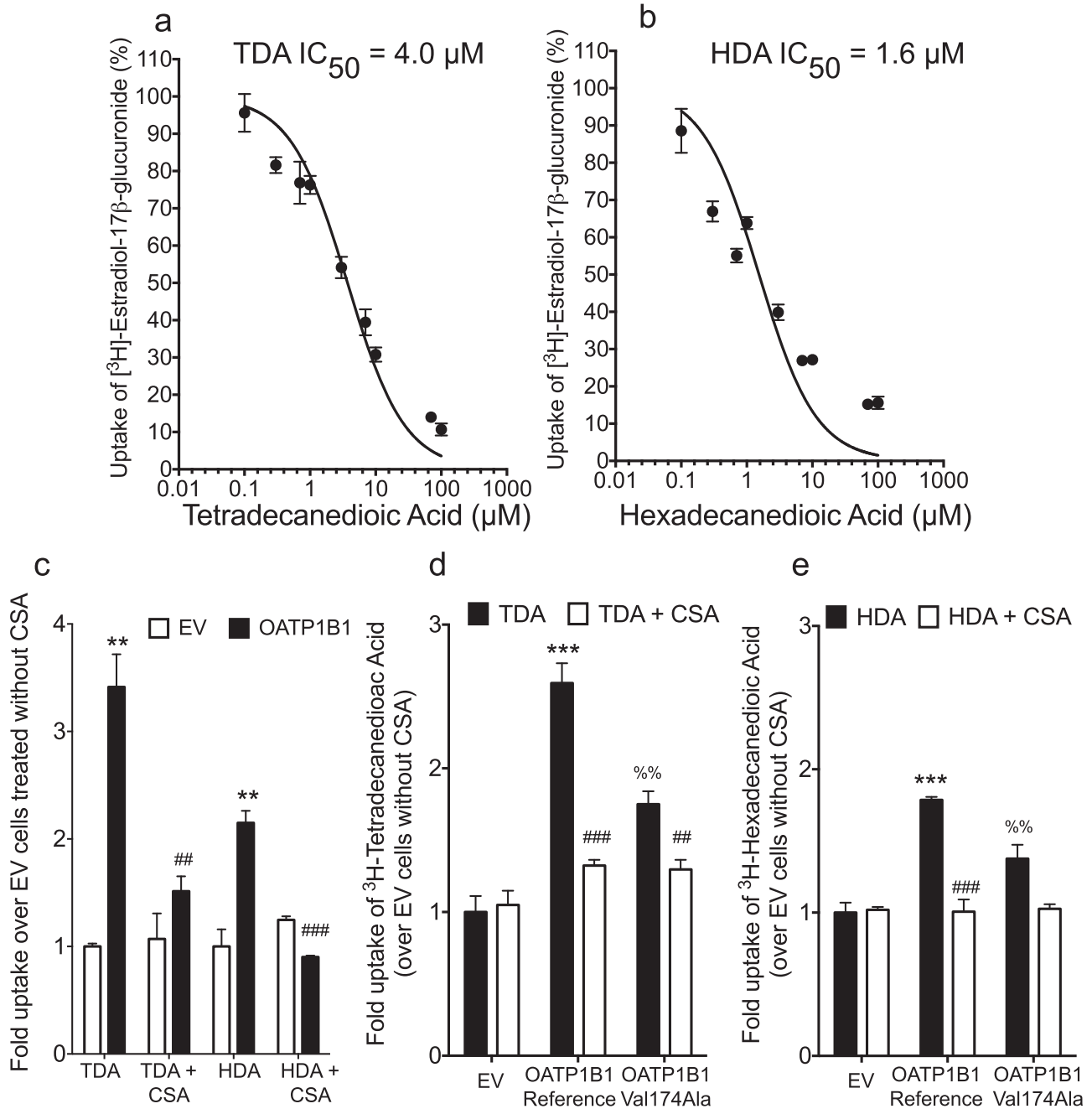

Figure 3 Inhibition (**a** and **b**) and uptake (**c**) studies of tetradecanedioate acid (TDA) and hexadecanedioate acid (HDA) in HEK293 cells stably expressing organic anion transporter OATP1B1. Uptake of ^{3}H-TDA (**d**) or ^{3}H-HDA (**e**) with or without cyclosporine (CSA; 10 μM) in HEK293 cells stably expressing OATP1B1 reference or OATP1B1-Val174Ala. (**a** and **b**) Representative curves of the effects of TDA and HDA on the uptake of ^{3}H-estradiol-17β-glucuronide (trace amount) in stably transfected HEK293 cell lines overexpressing OATP1B1. Concentrations of TDA and HDA ranged from 0.1 μM to 100 μM. (**c**) HEK293 Flp-In cells, stably transfected with a plasmid containing empty vector (EV) or cDNA of OATP1B1 were incubated with a trace amount of ^{3}H-TDA or ^{3}H-HDA with or without 10 μM CSA. Cells were incubated with the HBSS buffer containing the substrate with or without the inhibitor for 4 min. (**d** and **e**) Cells were incubated with the HBSS buffer containing the substrate with or without the inhibitor, CSA, for 4 minutes. A trace amount of ^{3}H-TDA (specific activity: 1.1 Ci/mmol) or ^{3}H-HDA (specific activity: 89.8 Ci/mmol) was used (1:5,000 dilution). Final concentrations of ^{3}H-TDA and ^{3}H-HDA that were used in the uptake studies were 180 nM and 12 nM, respectively. Fold changes are normalized to EV control uptake (without inhibitor). Results shown are the mean ± SD for a representative experiment, performed in triplicate. The experiments were repeated in three independent experiments with similar trends and results. $^{***}P \leq 0.0001$; $^{**}P \leq 0.001$ (between EV and OATP1B1); $^{\#\#\#}P \leq 0.0001$; $^{\#\#}P \leq 0.001$ (between OATP1B1 without inhibitor and with inhibitor); $^{\%\%}P \leq 0.01$ (between OATP1B1 reference (Val174) and OATP1B1 Ala174).

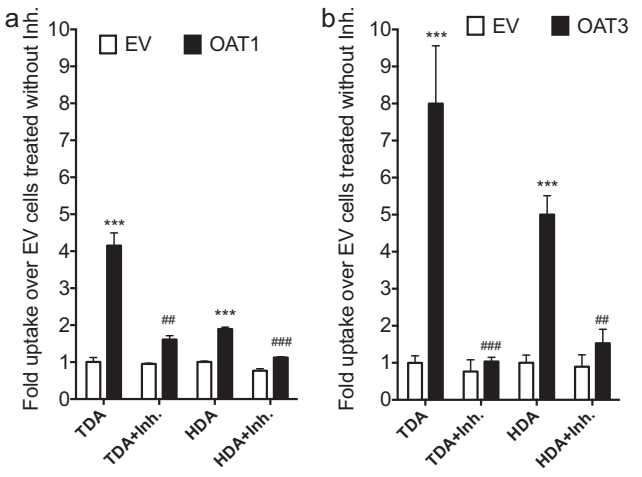

Figure 4 Uptake of ^{3}H-tetradecanedioate acid (TDA) or ^{3}H-hexadecanedioate acid (HDA) in HEK293 cells stably expressing (**a**) organic anion transporter OAT1 and (**b**) OAT3. Inhibitor (Inh.) used for each transporter was probenecid (100 μM for OAT1 and OAT3). Cells were incubated with the HBSS buffer containing the tritiated substrate with or without the inhibitor for 2 minutes. Trace amounts of ^{3}H-TDA (specific activity: 1.1 Ci/mmol) or ^{3}H-HDA (specific activity: 89.8 Ci/mmol) were used (1:5,000 dilution). Final concentrations of ^{3}H-TDA and ^{3}H-HDA that were used in the uptake studies were 180 nM and 12 nM, respectively. Fold changes are normalized to empty vector (EV) control uptake (without inhibitor). Results shown are the mean ± SD for a representative experiment, performed in triplicate. The experiments were repeated in three independent experiments with similar significance and trend. $^{***}P \leq 0.0001$ (between EV and OAT1 or OAT3); $^{\#\#\#}P \leq 0.0001$; $^{\#\#}P \leq 0.001$ (between OAT1 or OAT3 without inhibitor and with inhibitor).

TDA and HDA were then tested as substrates of OAT1 and OAT3 by quantifying uptake in transfected cells. Both TDA and HDA were significantly taken up in OAT1 and OAT3 transfected cell and this was inhibited by probenecid (**Figure 4a and 4b**). Notably, SNPs in OAT1 and OAT3 did show weak associations with the fatty acid dicarboxylates (*P* values ranged from 0.007 to 0.02; **Supplementary Table S5**).

DISCUSSION

The importance of DDIs mediated by transporters has been well recognized and, as a result, regulatory agencies from the United States, Europe, and Japan have issued guidelines to drug developers that rely primarily on *in vitro* transporter assays to inform decisions to conduct clinical DDI studies. Transporter assays are mechanistic and provide considerable insight into drug transporter interactions. However, *in vitro* drug transporter interactions may not be clinically relevant if the interaction of the compound with the transporter is not sufficiently potent.[6,24,25] Therefore, other tools are needed to complement these assays to more reliably predict potential clinical DDIs.

Although previous studies have used various methods in isolation, such as clinical DDI studies in humans[8,26] and studies in animals (e.g., monkeys and knockout mice[21,27,28]) to discover biomarkers, in this study, we used a multitiered approach combining human genetic and metabolomic studies with *in vitro* methods to discover endogenous metabolites that could serve as biomarkers of the widely studied hepatic organic anion transporter, OATP1B1. The beauty of such biomarkers is that they can be used from as early as first-in-human studies to suggest potential *in vivo* DDIs before conducting costly DDI studies. As the metabolites are measured from *in vivo* serum, plasma, or urine samples, they may be more accurate in predicting DDIs than *in vitro* studies of drug-transporter interactions. Ideally, an endogenous biomarker for a drug transporter should be stable in the collected matrix, selective for the transporter of interest, quantitatively associated with clinical DDIs involving the transporter, and not be affected by normal or disease conditions. Measurement of the biomarker should be quantitative, accurate, selective, sensitive, and reproducible. Our major findings were that: (a) many metabolites were significantly associated with genetic variants in various SLC transporters; (b) 12 metabolites were significantly associated with *SLCO1B1* (OATP1B1) in both the GWAS and the CSA DDI study; (c) among six metabolites tested *in vitro*, we identified two novel substrates of OATP1B1; and (d) the six metabolites also interacted with other xenobiotic enzymes and transporters.

Many metabolites were associated with genetic variants in various SLC transporters

Data collected from the metabolomics GWAS server revealed that many serum metabolites were strongly associated with SLC transporter variants at genome-wide significance ($P < 5 \times 10^{-8}$). Some of these metabolites were documented substrates of the transporters and replicated in other genome-wide association studies of human metabolites (**Supplementary Table S2**). Importantly, several potential new substrates were identified, such as histidine, which was associated with the uric acid transporter, *SLC2A9*. Further studies are clearly needed to determine whether these metabolites are substrates of the transporters. In addition, future studies are needed to validate if these metabolites (**Supplementary Table S1**, **Supplementary Table S2**) can be candidate probes for use clinically to identify drugs likely to cause a potential transporter-mediated DDI. This would be of particular importance for transporters playing a role in the pharmacokinetics of many prescription drugs (e.g., OATP1B1, OAT1, organic cation transporter 2, and multidrug and toxin extrusion protein 1). Such biomarkers could also be useful to investigate variation in pharmacokinetics due to genetic variants in drug transporters.

Twelve metabolites were significantly associated with *SLCO1B1* (OATP1B1) in both the GWAS and the CSA DDI study

OATP1B1, a highly expressed transporter in the human liver, is known to transport a variety of endogenous metabolites, including bile acids, steroids, bilirubin, eicosanoids, and thyroid hormones (see reviews[16,29]). In this study, several of the known endogenous metabolites of OATP1B1 were found to significantly associate with the reduced function variant of OATP1B1 (rs4149056; see **Supplementary Table S3**). Notably, the most significant association among all SLC transporters was the

association of X-11529 (glycochenodeoxycholate glucuronide) levels with OATP1B1-Val174Ala ($P = 5.5 \times 10^{-315}$). In fact, conjugates of bile acids represented 4 of the 12 metabolites significantly associated with OATP1B1 genetic variants. These conjugates also increased following administration of CSA. Consistent with our results, a previous study has shown that genetic variants in *SLCO1B1* associated with plasma levels of glycochenodeoxycholate[30] as well as bilirubin glucuronide conjugates.[19] However, in the GWAS, there were significant associations of genetic variants in genes encoding other transporters (OATP1B3, OATP1A2, MRP2, and BSEP) in addition to OATP1B1 with conjugated bile acids (**Supplementary Table S5**) consistent with multiple interactions between hepatic and intestinal transporters and sulfate and glucuronide conjugates of bile acids.[31] Because CSA is a nonspecific OATP1B1 inhibitor with potent inhibitory effects on transporters, such as OATP1B3, MRP2, NTCP, and BSEP[32] (**Table 2**), observed effects in the clinical study could be a result of inhibition of other transporters as well. Even with this complexity, use of these methods helped to identify potential selective metabolites as substrates of OATP and OAT. It is worth mentioning that the metabolomic platform that was used in our study did not detect some metabolites that have previously been reported as potential OATP1B1 biomarkers (e.g., coproporphyrins I and III[28,33]). This may be due to differences in the methodologies used for metabolite quantification.

Fatty acid dicarboxylates are novel substrates of OATP1B1

Among the six metabolites that were selected for *in vitro* studies as inhibitors, the bile acids inhibited other OATPs and other bile acid transporters, BSEP, NTCP, and MRP2 (**Table 2**). Although not evaluated in this study, the conjugated bile acids and steroids are likely to interact with OATP1B1 and other transporters (e.g., OATP1B3, OAT3, NTCP, ABCC2, ABCG2, or ABCB11) due to evidence in the literature indicating that glucuronide and sulfated conjugates interact with these transporters.[34,35] To our knowledge, this study for the first time demonstrates that fatty acid dicarboxylates, such as TDA and HDA, respectively, are substrates of OATP1B1 (**Figure 3**, **Table 1**). The results suggest that these may be useful biomarkers of OATP1B1. Interestingly, we noted that TDA (**Supplementary Figure S3**) as well as HDA and 8 of the 10 other metabolites significantly correlated with pravastatin area under the curve (**Supplementary Table S6**) in 16 individuals with genotype TT and TC genotypes for rs4149056 (OATP1B1-Val174Ala). TDA and HDA had a small dynamic range whereas other metabolites (e.g., X-11529), had larger dynamic ranges; however, TDA, HDA, as well as X-11529 correlated with pravastatin area under the curve. These data suggest that the metabolites may potentially serve as quantitative biomarkers of OATP1B1 activity, although further work is clearly needed.

The metabolites were found to also associate with other xenobiotic enzymes and transporters

Many drugs are known to interact with several metabolizing enzymes and transporters.[36] Similarly, in these studies, we noticed that several of the metabolites that were associated at genome-wide level significance with *SLCO1B1* were significantly associated with metabolizing enzymes. These enzymes included CYP4A11, which was associated with TDA and HDA levels, and SULT2A1, which was associated with X-13429, X-12850, and X-11440 levels (**Figure 2a**). This reflects the complexity of endogenous metabolites disposition and metabolism, and shows the difficulty in identifying biomarkers for a single enzyme or transporter.

In order to eliminate the possibility that TDA and HDA levels were elevated upon CSA administration in the clinical DDI study, we evaluated whether CSA inhibit CYP4A11. In our study, we showed that CSA did not inhibit CYP4A11 (**Supplementary Figure S4**). However, the CYP4A11 assay shown in **Supplementary Figure S4** may suggest that fatty acid dicarboxylic acids are substrates or products of CYP4A11. This is consistent with the information from literature that CYP4A11 has been implicated in the production of dicarboxylic acid.[37]

Notably, metabolites may interact with multiple transporters. In our study, we showed that TDA and HDA, which are substrates of OATP1B1, are also substrates of the renal organic anion transporters, OAT1 and OAT3. Although the results suggest that these two fatty acid metabolites may be potential biomarkers of these transporters, there is currently no information about the affinity of the metabolites for OATP1B1, OAT1, and OAT3. To our knowledge, there is no published information about the plasma concentrations of these metabolites, which together with kinetic information is needed to validate the *in vivo* relevance of OATs as determinants of the disposition of these metabolites. Relative blood or urine concentrations of DCA or conjugates, and factors that modulate them (e.g., disease, nutritional status, drug intake) are not well defined, with the exception that DCA levels are elevated in patients with Reye syndrome.[38] Use of TDA or HDA as endogenous biomarkers of OATP1B1 alone or in combination with the OAT1 and OAT3 is intriguing and calls for further evaluation.

Future directions

Overall, from our multitiered approach, we identified endogenous metabolites that interact with OATP1B1, including previously unknown substrates of the transporter. These biomarkers may potentially be used as probes to determine whether new drugs under development are likely perpetrators of OATP1B1-mediated DDIs. The use of sensitive and specific endogenous biomarkers may help drug developers avoid unnecessary clinical DDI studies predicted from current *in vitro* methods.[1] However, additional clinical studies are needed to validate these metabolites (conjugated bile acids, TDA, HDA, and conjugated steroids) and the specificity of the biomarkers needs to be ascertained. In particular, are the biomarkers specific for OATP1B1 or do they also serve as biomarkers for other OATs, such as OAT1 and OAT3. Other factors, such as disease, diet, and ethnicity, which may affect the levels of these biomarkers, should be characterized. Finally, the sensitivity of the biomarkers (e.g., the magnitude of change in the biomarker levels that can be detected in the presence of strong, moderate, or weak inhibitors) and their quantitative relationship with changes in the levels of known OATP1B1

model substrates (e.g., pravastatin, rosuvastatin) need to be established.

MATERIALS AND METHODS

Genome-wide approach to identify endogenous metabolites for SLC transporters

Several genome-wide studies have been published to identify the genetic factors that associate with human metabolites levels.[11,12,39–42] In general, samples from these studies were collected from fasting healthy volunteers and the metabolites were measured using different approaches. Shin *et al*.[11] have published a meta-analysis, which includes 7,824 adult individuals from two European populations and the associations of their genetic variants with over 400 metabolites in blood samples. The complete datasets are available from this link, http://mips.helmholtz-muenchen.de/proj/GWAS/gwas/gwas_server/shin_et_al.metal.out.tar.gz. These datasets were used as our primary discovery of endogenous metabolites for SLC transporters. The SNPs for all 52 family members of the SLC transporters (total number = 390), including 25,000 bp up and downstream of the transcription start and stop sites and the levels for all 529 metabolites were extracted from the datasets. Metabolites and SLC transporters with associations (P values $< 10^{-5}$) were extracted for exploration and hypothesis generation.

Clinical samples for cyclosporine/pravastatin coadministration in healthy subjects

For the clinical metabolomic study, we used plasma obtained from a DDI study of CSA and pravastatin.[43] In particular, plasma samples in 20 healthy volunteers collected 30 minutes after the second dose of CSA (100 mg) or placebo were used for metabolite measurements. Volunteers were genotyped for rs4149056 (Val174Ala), and included 10 who were homozygous for the reference allele, 7 who were heterozygous, and 3 who were homozygous for the variant allele. For the correlative analysis, we included nine and seven homozygous for the reference allele and heterozygous for the variant allele of rs4149056 respectively, as one subject (who is homozygous for the reference allele) did not achieve measureable pravastatin levels and, therefore, was omitted from the analysis. Further information is available in **Supplementary Information**.

Quantification of metabolites in the clinical samples for cyclosporine/pravastatin coadministration

Metabolites were measured in the plasma samples using the Metabolon platform (The DiscoveryHD4 Platform), which represents a comprehensive and unbiased way of identifying and quantifying metabolites. Plasma metabolite levels in the same individuals in both cyclosporine and placebo were determined using the platform. Extracted plasma samples were processed via three ultra-high-performance liquid chromatography/tandem accurate mass spectrometry methods and an additional gas chromatography/mass spectrometry method. Further information is available in the **Supplementary Information**.

Chemicals and reagents

TDA, HDA, taurolitocholate-3-sulfate, glycochenodeoxycholate, glycodeoxycholate, and 1,5-AG were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]-TDA was custom synthesized by Moravek Biochemicals (Brea, CA). The following radiolabeled compounds were used as model substrates for specific transporters: [^{3}H]-esterone sulfate (Perkin Elmer); [^{3}H]-para-aminohippurate (American Radiolabeled Chemicals); [^{3}H]- guanosine 3′,5′-cyclic phosphate (Moravek); [^{3}H]-cholecystokinin octapeptide (Perkin Elmer); [^{3}H]-taurocholic acid (Perkin Elmer); and [^{3}H]-estradiol-17β-D-glucuronide (Perkin Elmer). In addition, [^{3}H]-HDA and [^{3}H]-1,5-AG were customized synthesized by Sanofi-Aventis (Frankfurt, Germany) see **Supplementary Information**.

Cell culture and *in vitro* transport and inhibition studies

Cells stably expressing EV, OATP1B1,[44] OATP1B3,[45] OAT1,[46] OAT2,[47] and OAT3[48] were cultured in the following conditions. Stably transfected HEK-293 Flp-In cells were maintained in Dulbecco's Modified eagle medium H-21 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 150 μg/mL hygromycin B. In some transporter studies (OATP1A2, OATP1B1, and NTCP), HEK293T/17 (ATCC) were used for transient transfection and were cultured in the above medium but without the hygromycin B (selection antibiotic). See **Supplementary Information** for more information about the radiolabeled compounds used as substrates for specific transporters in the inhibition studies.

Statistical analysis

The P values were determined using paired nonparametric t tests to analyze the differences in metabolite levels in plasma from placebo-treated vs. CSA-treated healthy volunteers. An estimated false discovery rate (q value) is calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. False discovery rate adjustment is an acceptable and alternative multiple test adjustment and particularly relevant for exploratory and data-driven testing. For exploratory analysis of our clinical DDI study with CSA, q value <0.2 was considered significant. Unless specified, data are expressed as mean ± SD. For *in vitro* transporter studies, statistical analyses were performed by unpaired t tests to determine significant differences between: (i) cells transfected with EV or transfected with vector containing transporter cDNA; and (ii) cells treated with no inhibitor or with inhibitor of specific transporter. The data were analyzed using GraphPad Prism 6.0 (La Jolla, CA). A P value < 0.05 was considered statistically significant.

Additional Supporting Information may be found in the online version of this article.

ACKNOWLEDGMENTS

We would like to acknowledge the funding sources from Sanofi-Aventis and National Institutes of Health grants for: K.M.G., D.L.K, S.W.Y, and A.Z. (U19GM061390), C.-H.H. (U01FD004979), X.M.L and S.G (T32 GM007175), and M.M.G. (T32 GM07546). In addition, we would like to acknowledge the following collaborators for their contributions to: computational and laboratory experiments in Sanofi-Aventis Deutschland GmbH (Dr. Hans Matter, Dr. Christian Buning, Thomas Koose, Marion Schmidt, and Alexandra Kautz), synthesis of radioligands in Sanofi-Aventis Deutschland GmbH (Volker Derdau, Jens Atzrodt); metabolomics profiling in Metabolon (Jeff Buckthal and Robert P. Mohney); protocol preparation and volunteer recruitment (Dr. Richard A. Castro from the University of California – San Francisco); facilities for clinical studies (University of California – San Francisco, Clinical & Translational Science Institute (CTSI) (grant UL1 RR024131); and computational code writing (Doug Stryke and Danny S. Park from University of California – San Francisco).

AUTHOR CONTRIBUTIONS

K.M.G., S.W.Y., M.G., J.M.K., W.B., and D.L.K. wrote the manuscript. K.M.G., S.W.Y., M.G., M.G., C.-H.H., D.W., X.L., S.G., J.M.K., A.Z., K.M., W.B., and D.L.K. designed the research. S.W.Y., M.G., C.-H.H., D.W., X.L., S.G., A.C., A.Z., W.B., and D.L.K. performed the research. K.M.G., S.W.Y., M.G., D.W., X.L., S.G., J.M.K., A.Z., K.M., W.B., and D.L.K. analyzed the data. K.M.G., K.M., W.B., and D.L.K. contributed new reagents/analytical tools.

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